ATTENUATED INFLUENZA VIRUSES

The present invention relates to modified viruses, in particular attenuated influenza viruses which may be employed as an influenza virus vaccine. Modified viruses of the invention also include recombinant attenuated influenza viruses suitable for use as viral vectors for expression of heterologous sequences in target cells.

Influenza remains a constant worldwide threat to human health. While inactivated influenza virus vaccines have been available for many years, such vaccines provide only limited protection. Previous efforts to provide a safe, live attenuated influenza vaccine have focussed primarily on cold-adapted influenza viruses. Thus, attenuated influenza viruses have previously been obtained by extensively passaging influenza virus at low temperatures. As a result of adaptation to growth at low temperature, influenza viruses which have lost their ability to replicate at higher temperatures (about 39°C) are obtained. The replication of such cold-adapted (CA) viruses is only slightly restricted in the cooler upper respiratory tract, but highly restricted in the warmer lower respiratory tract, the major site of disease-associated pathology. Sequence comparisons between wild-type and CA influenza viruses have revealed both silent mutations and non-silent mutations leading to amino acid changes in the coding regions of several gene segments. Most amino acid changes were found to be the result of point mutations. The genetic instability of point mutations, and the level of immunogenicity of CA influenza viruses, remain as perceived potential problems in use of CA influenza viruses as vaccines for worldwide general use.

Another approach to obtaining attenuated influenza viruses which has been investigated is the construction of chimeric influenza viruses in which a non-coding region of an influenza virus genomic segment is substituted by a non-coding region from a genomic segment of an influenza virus of a different type. Such attenuated chimeric A/B influenza viruses are discussed, for example , in Muster *et al.*, Proc. Natl. Acad. Sci. USA (1991) 88, 5177-5181, Luo *et al.*, J. Virology (1992) 66, 4679-4685 and Bergmann and Muster, J. General Virology (1995) 76 3211-3215.

Three types of influenza virus are known designated as types A, B and C. Each of these types has many strains. The genome of an influenza virus is a segmented genome consisting of a number of negative sense RNAs (8 in the case of types A and B and 7 in the case of type C), which encode (in the case of type A) 10 polypeptides: the RNA-directed RNA polymerase proteins (PB1, PB2 and PA) and nucleoprotein (NP) which form the nucleocapsid, the matrix proteins (M1, M2), two surface glycoproteins which project from the lipoprotein envelope (hemagglutinin (HA) and neuraminidase (NA)) and the non-structural proteins NS1 and NS2. The majority of the genomic RNA segments are monocistronic. Thus, in the case of influenza virus of type A, 6 of the 8 genomic RNA segments are monocistronic and encode HA, NA, NP and the viral polymerase proteins, PB1, PB2 and PA.

During the replication cycle of an influenza virus, the viral genome (vRNA) is transcribed into mRNA and replicated into complementary RNA (cRNA) molecules, which in turn are used as templates for vRNA synthesis. These processes are known to be catalyzed by the viral polymerase complex consisting of three subunits formed by the PB1, PB2 and PA polypeptides. mRNA synthesis is initiated by capped RNA primers, which are cleaved from host cell mRNA by an endonuclease associated with the viral polymerase complex. The synthesis of mRNA is prematurely terminated at a run of uridines, in the case of an influenza A virus 16 or 17 nucleotides away from the 5' end of the vRNA template, and subsequently a poly(A) tail is added. On the other hand, cRNA synthesis is believed to be initiated in the absence of primer resulting in full-length precise copies of the vRNA segments. The nucleoprotein has been implicated as a switching factor, which acts as an antiterminator during cRNA synthesis.

Influenza vRNA segments may be prepared *in vitro* by transcription from plasmid DNA and mixed with viral polymerase proteins and nucleoprotein to form ribonucleoprotein complexes (RNPs) having all the components necessary for transcription and replication. Such RNPs can be incorporated into viable influenza virus particles in cell packaging systems, e.g. employing a helper virus.

The development of RNP reconstitution and transfection systems has permitted detailed characterization of the RNA signals in influenza A vRNAs

involved in the regulation of transcription initiation, termination, and polyadenylation (4, 20-22, 25, 32, 34). All these signals are known to reside in the terminal sequences of vRNA segments (19). The 5' and 3' ends contain 13 and 12 conserved nucleotides respectively, which have the ability to form a partially double-stranded panhandle/RNA-fork or corkscrew structure (6, 7, 13). Initial *in vitro* transcription studies with model RNA templates implied that vRNA and cRNA promoters were located exclusively in the 3' terminal sequences (25, 32) and that the panhandle had no apparent role in the initiation of transcription *in vitro*. However, detailed mutagenesis studies of the terminal sequences subsequently showed that the 5' end forms an integral part of the promoter. These findings were based on binding experiments of the RNA polymerase to the putative promoter RNA (7, 33) and, more importantly, on *in vitro* transcription studies with mutant model template RNAs (7, 8, 28). In addition, activation of the viral polymerase-associated endonuclease requires interaction of the polymerase complex with the 5' as well as the 3' terminal sequences of vRNA segments (11).

The postulated double-stranded region of the promoter of an influenza A vRNA segment is now recognised to consist of 5 to 8 base-pairs. The first 3 base-pairs, those formed by nucleotides 11' to 13' at the 5' end and nucleotides 10 to 12 at the 3' end, are strictly conserved among different vRNA segments of all influenza A viruses. Sequencing studies have shown that the 3' and 5' non-coding terminal sequences of influenza B and C vRNA segments are also highly conserved and show partial inverted complementarity (36, 37). Consequently, it is believed that the capability of base-pairing of nucleotides of the non-coding regions to form a panhandle structure is important for proper functioning of all influenza vRNAs. The term duplex region of an influenza vRNA segment as used hereinafter will be understood to refer to the region which is formed by such base-pairing.

Kim *et al.* (14) have previously used a choloramphenicol acetyltransferase (CAT) reporter gene construct in which negative sense CAT RNA is flanked by the non-coding sequences of an influenza A virus NS gene to determine the effect of mutations in the postulated duplex promoter region on CAT expression in Madin-Darby bovine kidney (MDBK) cells. Negative-sense CAT RNA constructs

were incorporated into RNP complexes, which were then used to transfect monolayers of MDBK cells infected with a helper influenza virus and CAT activity assayed. Using this model system, single mutations of the conserved residues at positions 11 and 12 of the 3' terminus and at positions 12' and 13' of the 5' terminus of the CAT gene construct were found to abolish or virtually abolish CAT activity. The introduction of second complementary mutations into such constructs so as to restore the capability for Watson-Crick base-pairing was found, however, to partially restore CAT activity. Thus, the constructs with the base-pair substitutions of U12-A13' for C12-G13' and A11-U12' for C11-G12' were found to express CAT at 31% and 22% respectively compared to the control construct with wild-type influenza A gene non-coding regions.

The same CAT reporter gene system was also used to investigate the effect of mutations of the U10-A11' base-pair. Single mutations, U10 to G10 and A11' to C11', significantly decreased CAT activity, but both mutants exhibited detectable activity. A combination of the two mutations to introduce a G10-C11' base-pair did not give improved CAT activity. It was therefore suggested that the properties of the base-pair at positions 10-11' might be different from those at positions 11-12' and 12-13'.

Such experiments merely test the effect of influenza vRNA duplex region mutations on the expression of a heterologous CAT reporter gene in cultured human cells. It is not possible to predict from such studies whether mutations which allow some CAT activity will, when incorporated into an influenza vRNA genomic fragment, permit rescue of that fragment into a viable virus. Equally, it is not possible to predict, even if such mutations give rise to viable virus, whether such viruses will be attenuated. Indeed, this is supported by the finding of the inventors that the base-pair substitution of C12-G13' by U12-A13' in the NA gene vRNA segment of an influenza A virus can be rescued into a viable influenza A virus which does not show significant attenuation on MDBK cells (see the Examples).

In contrast, it has now been established that substitution of A for C and U for G at position 11-12' in the duplex region of the NA-specific vRNA of an influenza A virus does lead to attenuation on MDBK cells and also other cell types in culture. It

has also been shown that influenza A virus with the same base pair substitution is attenuated *in vivo* and can give rise to protective immunity against wild-type influenza A virus. Evidence suggests that such attenuation arises from reduced polyadenylation of the NA-specific mRNA. Base-pair substitution in the duplex region of a vRNA segment is thus proposed as a new general strategy for achieving attenuation of influenza viruses. Such base-pair substitution can be selected by application of known rescue systems for incorporating genetically-engineered influenza vRNA segments into viable influenza viruses as further discussed below.

In one aspect, the present invention thus provides an attenuated influenza virus carrying a genomic nucleic acid segment which comprises 5' and 3' non-coding regions providing a mutated duplex region of an influenza virus RNA genomic segment operably-linked to a protein coding sequence for an influenza viral protein or functional modification of said protein, wherein said duplex region is a non-chimeric duplex region, but has at least one base-pair substitution such that expression of the said protein-coding sequence in cells infected by the said virus is reduced to give an attenuated phenotype.

Mutated duplex region of an influenza virus RNA genomic segment will be understood to exclude any native influenza virus vRNA duplex region derived from a vRNA of a wild-type influenza virus of a different type.

The term "cells" in this context may encompass human and/or animals cells *in vivo* normally infected by influenza viruses. For the purpose of selection of attenuated viruses of the invention, the same term will be understood to refer to cells of a single cell type or more than one type, e.g. cultured human or non-human animal cells of one or more than one type. They may be *in vivo* cells, e.g. cells of an animal model. Cultured cells which may prove useful in the selection of attenuated viruses of the invention *in vitro* include one or more of MDBK cells, Madin-Darby canine kidney (MDCK) cells and Vero (African green monkey kidney) cells.

While an attenuated virus of the invention may have a single base-pair substitution in the duplex non-coding region of a genomic segment, it will be appreciated that such a virus may have more than one such substitution, either on the same genomic segment or different genomic segments, e.g. 2 base pair substitutions in the same genomic segment duplex region. The duplex base-pair substitution(s)

will desirably result in some, e.g. at least about one log, reduction in plaque titre compared to the parent wild-type virus on MDBK cells. The duplex base-pair substitution(s) will desirably provide an attenuated virus exhibiting some, e.g. at least about one log, more preferably at least about 3 to 4 log, reduction of plaque titre on MDCK cells and Vero cells compared to the parent wild-type virus. An attenuated virus of the invention may, for example, exhibit as much as about 5 log reduction of plaque titre compared to the parent wild-type virus on Vero cells arising from the vRNA non-coding region base substitutions. Such an attenuated virus is exemplified by influenza A/WSN/33 having an NA-specific vRNA segment incorporating the base-pair substitution A11-U12' for C-G at position 11-12' of the duplex region and additionally having the base-pair substitution G10-C11' for U10-A11' (mutant D1/2 referred to in the examples). Other influenza A viruses incorporating the same base-pair substitutions, either in the NA-specific vRNA segment or a vRNA segment encoding another influenza virus protein, also exemplify the invention.

As indicated above, attenuated viruses of the invention also include influenza A/WSN/33 having the single base-pair substitution A11-U12' in the NA-specific vRNA segment (mutant D2 referred to in the examples) and other influenza A viruses having the same base-pair substitution in the NA-specific vRNA segment or another viral protein-encoding vRNA segment. Thus, in one embodiment the present invention provides an attenuated influenza virus of type A carrying a mutated influenza A virus genomic RNA segment having the mutation C to A at position 11 from the 3' terminus of the native parent segment and the mutation G to U at position 12' from the 5' terminus of the native parent segment, or functionally equivalent substitutions such as modified base substitutions at the same positions, so as to provide an attenuating base-pair substitution in the non-coding duplex region. Additionally, in a further embodiment, the present invention provides such an attenuated virus of type A which in the same vRNA segment has the mutation U to G at position 10 from the 3' terminus of the native parent segment and the mutation A to C at position 11' from the 5' terminus of the native parent segment, or functionally equivalent substitutions at the same positions, so as to provide an additional base-pair substitution in the non-coding duplex region. Such a virus may be a wild-type virus

which has been attenuated by introduction of one or more base-pair substitutions as above into the non-coding duplex region, or a recombinant attenuated virus carrying a heterologous coding sequence as further discussed below. Desirably, for example, the attenuating base-pair substitution(s) will be introduced into the genomic nucleic acid segment encoding NA or a functional modification of that surface glycoprotein.

Although the invention is further illustrated hereinafter with particular reference to influenza A/WSN/33, the invention is not confined to influenza viruses of the A-type. Functionally equivalent mutations to the D2 or D1/2 mutations, i.e. attenuating base-pair substitutions, in viruses of the B and C types may be analogously identified by reference to available sequence information and application of known rescue systems applicable to any genetically-engineered influenza vRNA segment suitable for providing the characteristic of attenuation to a complete influenza virus.

Thus, a further embodiment of the invention, is an influenza virus of type B carrying a mutated influenza B virus genomic RNA segment, e.g. NA-encoding segment, having an attenuating base-pair substitution in the non-coding duplex region at a functionally homologous position to the base-pair substitution in influenza A/WSN/33 designated above as D2. The invention also extends to influenza viruses of type C carrying such a base-pair substitution in a mutated influenza C virus genomic RNA segment, e.g. a mutated NA-encoding segment.

Brief Description of the Figures

Figure 1 is a representation of the conserved sequences of an influenza A virus vRNA in the panhandle/RNA-fork conformation (7, 13). Conserved base-pairs in the double-stranded region of the RNA-fork, involving both the 5' and 3' ends of the RNA segment, are boxed. Numbering of residues starts from the 3' end and from the 5' end. The 5' end numbers are distinguished by prime ('). Base-pairs in the conserved double-stranded region of the modified NA-encoding vRNA of the transfectant viruses designated D1, D2, D3 and D1/2 in the examples are shown. Changed base-pairs are highlighted.

Figure 2 shows growth curves of transfectant viruses on MDBK cells.

Confluent cells in 35 mm dishes were infected with wild-type influenza A/WSN/33 (wild-type; WT) virus, and with the transfectant D1, D2, D3 or D1/2 viruses at a multiplicity of infection (m.o.i.) of 0.01. At the indicated time points, infectious particles present in the media were titrated by plaque assay in MDBK cells. The presented values are averages from duplicate experiments.

Figure 3 shows the nucleotide sequence of the plasmid pT3NAm1 containing the full-length cDNA of the NA gene of influenza A/WSN/33 (positions 2412-3820) flanked by a unique BbsI restriction site at one end (position 2404) and a bacteriophage T3 RNA polymerase promoter at the other end (positions 3821-3836) in the background of the pUC19 cloning vector between the EcoR1 (position 2398) and Hind III (position 3837) restriction sites (9). This plasmid was employed to obtain the mutant versions of the NA-encoding vRNA of influenza A/WSN/33 present in the D1, D2, D3 and D1/2 viruses (see Example 1).

Figure 4 shows the time course of pathogenicity of wild-type, D1, D2, D3 and D1/2 viruses in mice when intranasally infected with 10^3 plaque-forming units (pfu) (see Example 13).

Figure 5 shows body weight following intranasal infection of mice with wild-type, D1, D2, D3 and D1/2 viruses at 10^3 pfu.

Figure 6 shows the time course of pathogenicity of wild-type, D1, D2, D3 and D1/2 viruses in mice when intranasally infected with $3x10^4$ pfu.

Figure 7 shows body weight following intranasal infection of mice with wild-type, D1, D2, D3 and D1/2 viruses at $3x10^4$ pfu.

Figure 8 shows the time course of pathogenicity of wild-type, D1, D2, D3 and D1/2 viruses in mice when intranasally infected with 10^6 pfu.

Figure 9 shows body weight following intranasal infection of mice with wild-type, D1, D2, D3 and D1/2 viruses at 10^6 pfu.

Figure 10 shows viral titres (log pfu per ml) on lungs of mice at 3 days (left) and 6 days (right) post-infection , following intranasal infection with wild-type (WT) and D1, D2, D3 and D1/2 viruses at 10^3 pfu (see Example 14).

Figure 11 shows body weight of D2-immunised mice (3 dose levels: 10^6, $3x10^4$ and 10^3 pfu) following challenge with 10^6 pfu wild-type virus (see Example

15).

Figure 12 shows body weight of D1/2-immunised mice (3 dose levels: 10^6, $3x10^4$ and 10^3 pfu) following challenge with 10^6 pfu of wild-type virus.

A nucleic acid segment of a virus of the invention incorporating an attenuating base-pair substitution as discussed above, and DNAs capable of transcription to provide such a nucleic acid, also constitute additional aspects of the invention. A nucleic acid of the invention may preferably correspond to a mutated native influenza virus RNA genomic segment having an appropriate attenuating base-pair substitution in the non-coding duplex region. Such an RNA may have additional modifications, for example, one or more additional nucleotides added at the 3' and/or 5' terminus or internally which do not destroy function. It may be a chimeric RNA.

A DNA capable of transcription *in vitro* to provide an RNA nucleic acid segment of the invention may be initially constructed in a plasmid by application of conventional techniques and isolated from that plasmid by restriction endonuclease digestion. As illustrated by plasmid pT3NAm1 referred to above, for this purpose a cDNA of a native influenza virus vRNA segment may be inserted into a plasmid flanked by an appropriate promoter and a restriction endonuclease site. The cDNA may then be subjected to site-directed mutagenesis by, for example, PCR-directed mutagenesis employing appropriate mutagenic primers to provide a sequence encoding the desired mutated vRNA segment for transcription. Alternatively, a genomic nucleic acid segment of the invention may be synthesized.

For preparation of an attenuated virus of the invention, a genomic nucleic acid segment having at least one attenuating base-pair substitution as defined above may be complexed *in vitro* with influenza viral polymerase proteins and nucleoprotein to form a RNP complex. Such RNP complexes, which constitute a still further aspect of the present invention, may be prepared in conventional manner as previously employed for incorporation of genetically-engineered influenza virus RNA genomic segments into RNA complexes for viral rescue in cells (4, 5, 38). RNP complexes of the invention may be transfected into cultured cells, e.g. MDBK

cells, MDCK cells or Vero cells, again using conventional techniques. Methods commonly employed for this purpose include DEAE-dextran transfection and electroporation (19, 39).

In yet another aspect, the present invention provides a method of preparing an attenuated influenza virus of the invention which comprises providing in a host cell the genomic nucleic acid segments for said virus under conditions whereby said segments are packaged into a viral particle. For this purpose, the genomic nucleic acid segments may be provided in the host cell by plasmids. Alternatively, RNP complexes of the invention as hereinbefore described may be transfected into host cells that have previously been infected with an influenza helper virus to complement the RNP complexes and enable selection of the desired attenuated viral particles. A number of helper virus-based cellular rescue systems for particular influenza virus genes have previously been described and have been reviewed by Muster and García-Sastre (56). Such gene specific rescue systems are briefly summarized below.

Helper virus based influenza gene rescue systems

Helper based rescue systems have been reported allowing the genetic manipulation of influenza A vRNAs for NA and HA surface antigens, the non-structural proteins, NP, PB2 polymerase protein and the M proteins.

NA gene specific rescue system

The most commonly employed helper virus based influenza gene rescue system is limited to the NA of influenza A/WSN/33 virus (4, 5). This method is based on the observation that only influenza viruses with an NA gene from influenza A/WSN/33 are able to grow on MDBK cells in the absence of trypsin. In this rescue system, the helper virus is a reassortant containing seven gene segments from influenza A/WSN/33 and a NA gene from a virus other than influenza A/WSN/33. Generally A/WSN-HK, which has an NA gene from influenza A/HK/8/68, is used as the helper virus. In this system, the NA gene of influenza A/WSN/33 is transfected into cells infected with the helper virus. The virus is then selected by growing on MDBK cells in the absence of exogenous proteases.

NA genes can also be rescued by using a NA-deficient mutant virus as a helper virus. Such a helper virus requires exogenous neuraminidase to grow in tissue culture. The NA-gene is transfected into cells infected with the helper virus. The virus is then selected by growing on cells in the absence of neuraminidase (43).

NS gene specific rescue system

A temperature-sensitive influenza virus with a defect in the NS1 protein is used as the helper virus of a NS gene specific rescue system. The NS gene segment carries two overlapping genes coding for the NS1 and NS2 proteins. This rescue system allows the rescue of a NS gene segment encoding an NS1 protein which has activity at the non-permissive temperature. In this system, the NS gene segment which is to be rescued is transfected into cells infected with the temperature-sensitive virus. The virus with the transfected NS gene segment is selected by growing the virus at the non-permissive temperature as described by Enami *et al.* (40).

PB2 gene specific rescue system

A virus with an avian influenza A virus PB2 gene can be used as the helper virus in a PB2 gene specific rescue system. The avian influenza A virus PB2 gene restricts the replication of the helper virus in mammalian cells. Therefore, this rescue system can rescue a PB2 gene which allows replication of influenza virus in mammalian cells. The PB2 gene which is to be rescued is transfected into cells infected with the helper virus. The virus with the transfected PB2 gene is selected by growing the virus in mammalian cells. Subbarao *et al.* (41) have used such an avian influenza A virus PB2 gene based system to rescue the PB2 gene of wild-type influenza A/Ann Arbor/6/60 virus.

M gene specific rescue system

An amantidine-sensitive influenza virus carrying an M gene of influenza A/equine/Miami/1/63 virus can be used as a helper virus of an M gene specific rescue system. The rescue system allows the rescue of an M gene which confers amantidine resistance to a virus. In this system, the M gene which is to be rescued is

transfected into cells infected with the helper virus. The virus with the transfected M gene is selected by growing the virus in the presence of amantidine. Castrucci and Kawaoka (42) have used such an amantidine-sensitive M gene based system to rescue the M gene of influenza A/PR/8/34 virus.

Antibody-based rescue systems

These systems depend on the binding or non-binding of the transfectant virus to a particular antibody (5, 52). Such antibody is a neutralising antibody which binds to influenza virus and impairs its growth in tissue culture. The helper virus may, for example, carry a gene which encodes an influenza surface protein which displays the antibody epitope. This system can therefore be used to select for transfectant virus which does not carry such a gene, but which of course is viable. This type of rescue system thus allows the rescue of a gene encoding an influenza surface protein. The gene to be rescued is transfected into cells infected with the helper virus. The virus with the transfected gene is selected by growing the virus in the presence of the antibody. Such a system was used by Enami and Palese (5) to rescue a transfected synthetic HA segment.

NP gene specific rescue system

Li and coworkers (39) reported a reverse genetics system for the rescue of the influenza A virus nucleoprotein gene. In this system, a temperature-sensistive (ts) mutant ts56 is used as a helper virus. RNA complexes are reconstituted *in vivo* as described before (5) and are then introduced by electroporation into ts56 helper virus infected cells. Transfectant viruses with a rescued NP-encoding vRNA segment are selected at the non-permissive temperature by plaquing on MDBK cells.

Influenza B virus rescue system

Barclay and Palese (44) have additionally described the rescue of HA genes in an influenza B virus.

The preparation of an attenuated virus of the invention may alternatively be

achieved using the expression vector-based influenza gene rescue strategy developed by Pleschka *et al.* (45). In contrast to the RNP transfection system referred to above, this eliminates the need for purification of the viral NP and polymerase proteins which is required for *in vitro* reconstitution of RNP complexes. Expression vectors are co-transfected into host cells which will provide the NP and P proteins and also a genomic segment of the invention incorporating an attenuating base-pair mutation. In this case, RNP complexes of the invention are formed intracellularly. The cells may then be infected with an influenza helper virus as previously described to select for the required attenuated influenza virus .

An RNA complex of the invention may also be rescued in host cells into a viable attenuated virus by transfecting into the host cells additional complementing RNA complexes thereby eliminating the need for a helper virus. This may be achieved in accordance with the general rescue strategy for influenza virus genes more recently described by Enami (46). This strategy involves purifying RNPs from an appropriate influenza virus and treating the RNPs *in vitro* with RNase H in the presence of a cDNA which hybridizes to the influenza virus gene to be rescued. In this way specific digestion of that gene by the RNase H is achieved. The gene depleted RNPs are then co-transfected into cells with the RNP-complex containing the nucleic segment to provide the attenuating base-pair substitution. The cells are then overlaid with agar and transfectant attenuated viruses obtained by direct plaque formation. This strategy, unlike the above described helper virus-based gene rescue strategies, can be applied to any influenza gene from any influenza virus. It can thus be applied to obtain an attenuated virus or gene of the invention of any influenza type.

Since reversion of a base-pair mutation requires two specific mutations, attenuated influenza viruses of the invention are expected to be highly stable (see Example 12). Hence, such viruses may be particularly favoured for use as influenza virus vaccines.

As indicated above, a virus of the invention may additionally contain a heterologous coding sequence capable of being expressed in target cells. Such a heterologous coding sequence may encode an antigenic peptide or polypeptide

capable of stimulating an immune response (either an antibody response or a cell-mediated immune response) to a pathogenic agent. Representative examples of such pathogenic agents are viruses, e.g. other influenza viruses or non-influenza viruses such as HIV, bacteria, fungi, parasites, eg. malarial parasites, and disease-causing cells such as cancer cells.

Thus, in yet another aspect, the present invention provides a vaccine comprising a virus of the invention. Particularly preferred are such vaccines wherein the attenuated influenza virus acts as a combined vaccinating agent against more than one pathogenic agent, e.g. an influenza virus and a second pathogenic agent other than an influenza virus. Such vaccines may be formulated and administered in accordance with known methods for this purpose.

Thus in a still further aspect, the present invention provides a method of stimulating an immune response against an influenza virus, e.g. an influenza virus of Type A, either alone or together with stimulation of an immune response against one or more further pathogenic agents, which comprises administering in an immunising mode an attenuated influenza virus of the invention capable of inducing said immune response(s). Intranasal immunisation with an attenuated influenza virus of the invention may, for example, be preferred. Such immunisation may be carried out as illustrated by the immunisation studies with recombinant influenza viruses expressing an HIV-epitope reported by Muster *et al.* (49) and Ferko *et al.* (53) (see also Example 15). A suitable immunisation dose may be, for example, in the range of 10^3-10^9 pfu. Booster immunisations may be given following an initial immunisation with a virus having the same functional characteristics, but of a different subtype or type.

Methods for incorporating heterologous coding sequences into an influenza virus have previously been described, for example, in Published International Application WO91/03552 (Palese *et al.*) and are also reviewed by Muster and Garcia-Sastre in Textbook of Influenza 1998 (56). The heterologous coding sequence may be on a genomic segment incorporating an attenuating base-pair substitution or on a different genomic segment. It may be carried by an additional nucleic acid segment also incorporating a gene for an influenza viral protein to

provide selection pressure. It has previously been reported, for example, that an influenza virus can be constructed carrying at least 9 different vRNA segments (40).

Use of attenuated recombinant influenza viruses of the invention as vectors to express foreign antigens for vaccinating purposes is an attractive therapeutic strategy since:

(i) Antibodies to the different subtypes show little cross-reactivity. One drawback with the use of a virus as a vaccine is that an immune response will be produced to the virus. It is often desired that one or more booster immunisations comprising the same antigen are given after the initial immunisation. However, the immune response to the virus reduces the effectiveness of subsequent immunisations with the same virus. Since antibodies to different influenza subtypes show little cross-reactivity, subsequent immunisations with an influenza virus of a different subtype but which expresses the same antigen should overcome this effect.

(ii) Influenza viruses have been shown to induce strong cellular and humoral responses.

(iii) Influenza viruses have been shown to induce strong mucosal responses. Intranasal immunisation with influenza virus has been shown to induce long lasting responses in genital and intestinal mucosa.

(iv) Influenza viruses are non-integrating and non-oncogenic.

(v) As previously noted above, attenuated influenza viruses of the invention can be anticipated to be attenuation stable.

For vaccinating purpose, a heterologous coding sequence may be provided in an attenuated virus of the invention encoding an antigen of a pathogenic agent or a modification thereof capable of stimulating an immune response. The heterologous coding sequence may be inserted into a viral gene to provide a fusion protein which retains the function of the parent viral protein. One site which has previously been found to tolerate insertions of foreign antigens (epitope grafting) is the antigenic B site of HA. Antigenic site B of that surface protein consists of an exposed loop structure located on top of the protein and is known to be highly immunogenic. Manipulation of the HA gene of an influenza virus to insert a viral epitope in the HA

protein B site has previously been reported (see again the studies of Muster *et al.* reported in 49 and the studies of Li *et al.* reported in 48). The same strategy has also previously been employed by Rodrigues *et al.* to express B-cell epitopes derived from a malaria parasite (50). Heterologous coding sequences for an antigenic polypeptide may also, for example, be preferably inserted into an influenza virus NA gene. Strategies for epitope grafting into influenza viral proteins have also previously been described, for example, in WO91/03552.

Epitope grafting of a foreign sequence into an influenza virus protein may result in a non-functional chimeric viral protein and make the rescue of a viable transfectant virus impossible. A different strategy for expressing foreign sequences by recombinant influenza viruses, which may be applied to attenuated viruses of the present invention, involves the engineering of gene segments containing an additional open reading frame. A recombinant genomic segment may be constructed which provides an internal ribosome entry site for a heterologous coding sequence. This approach has previously been used, for example by García-Sastre *et al.* to obtain an influenza virus vRNA segment which encodes both a truncated form of gp41 of HIV and NA (9). Alternatively, a heterologous coding sequence may be fused in frame to a viral protein coding sequence to encode a chimeric polyprotein capable of autoproteolytic protease cleavage to give the viral protein and a desired second polypeptide, e.g. a viral antigen. This strategy has been shown by Percy *et al.* to be suitable for expressing non-influenza proteins up to 200 amino acids in length (51).

It will be appreciated that a recombinant attenuated virus of the invention may be employed as a vehicle for expression of heterologous coding sequences in target cells for a variety of therapeutic purposes in addition to vaccination. Such a recombinant virus may, for example, have a genomic segment encoding any of the following:

- a cytokine such as an interferon or an interleukin,
- a toxin,
- a palliative capable of inhibiting a function of a pathogenic agent either directly or indirectly, e.g. a viral protease inhibitor
- an enzyme capable of converting a compound with little or no

cytotoxicity to a cytotoxic compound, e.g. a viral enzyme such as Herpes simplex thymidine kinase capable of phosphorylating purine and pyrimidine analogues to active toxic forms,

- an antisense sequence,
- a ribozyme.

Sequences encoding such agents may be incorporated into an attenuated influenza virus of the invention by any of the techniques previously referred to above in connection with providing attenuated viruses of the invention expressing foreign epitopes.

A heterologous coding sequence in an attenuated recombinant virus of the invention may be under the control of a tissue-specific and/or event-specific promoter. A recombinant virus of the present invention may be employed for gene therapy.

A recombinant virus of the invention may be administered directly or used to infect cells *ex vivo* which are then administered to a patient.

Thus, in still further aspects, the present invention provides a pharmaceutical composition comprising a recombinant virus of the invention in combination with a pharmaceutically acceptable carrier or diluent for delivery of a heterologous coding sequence to target cells. It also provides *ex vivo* cells infected by a virus of the invention and such cells hosting a recombinant influenza virus of the invention formulated for administration with a pharmaceutically acceptable carrier or diluent. In yet another aspect, the present invention provides a method of delivering a heterologous coding sequence to cells which comprises infecting said cells with an attenuated recombinant influenza virus of the invention carrying said sequence.

Viruses of the invention may also find use as a helper virus to rescue genes which can substitute for the gene(s) affected by the attenuating mutation(s) to provide viruses showing increased growth on a selected cell type. For this purpose, an attenuated virus will preferably be chosen which exhibits at least about a 3-4 log, preferably at least about a 5 log, reduction in growth compared to the corresponding wild-type virus on one or more cell types. Thus, in yet another embodiment, the present invention provides use of a virus of the invention as a helper virus to rescue

an influenza virus genomic nucleic acid segment in cells, wherein viruses produced containing said segment are selected on the basis of increased growth compared with the helper virus on cells of a selected type. For example, an influenza A virus of the invention having an attenuating base-pair substitution in the non-coding duplex region of its NA-encoding vRNA may be usefully employed to rescue an NA-encoding vRNA or functional modification thereof derived from a second influenza A virus. A typical protocol for this purpose will comprise the steps of:

1. infecting cells with the helper virus,
2. transfection of an RNP complex containing the gene(s) to be rescued into the helper virus infected cells, and
3. selection of rescued viruses, either on the same cell type or a different cell type on which the helper virus shows increased attenuation.

The cell type in step 3 will be chosen such that only viruses which have acquired the transfected gene(s) are expected to grow to high titre.

For example, the D1/2 mutant version of influenza A/WSN/33 referred to above is particularly favoured as a helper virus for use to rescue NA genes originating from other influenza viruses of the A-type. In this case, MDBK cells may, for example, be initially infected with the D1/2 helper virus and Vero cells preferably used for selection of viruses carrying an NA gene containing vRNA without an attenuating mutation. The D2 mutant derived from influenza A/WSN/33 may similarly be employed.

Influenza A/WSN/33 is known to exhibit in mice neurovirulence associated with the surface antigen NA (54). For this reason, the attenuated modified versions of that virus referred to above are not regarded as suitable for direct vaccine use. However, by using, for example, the D1/2 mutant as a helper virus as above, NA vRNAs may be obtained for site-directed mutagenesis to construct alternative attenuated influenza A viruses according to the invention more suitable for therapeutic, e.g. vaccine, use.

The following examples illustrate the invention.

Example 1

Introduction of mutations into the duplex region of the NA-encoding vRNA of an influenza virus of type A

In order to produce NA-encoding viral genomic RNA with mutations in the 5' and 3' non-coding regions, plasmids were constructed which contained the corresponding cDNA with the desired mutations.

The starting plasmid for site-directed mutagenesis was pT3NAm1 (see Figure 3) which, as previously noted above, contains the full length cDNA of the NA gene of influenza A/WSN/33 virus (positions 2412-3820) flanked by a unique BbsI restriction site at one end (position 2404) and a bacteriophage T3 RNA polymerase promoter at the other end (positions 3821-3836) in the background of the pUC19 cloning vector between the EcoR1 (position 2398) and Hind III (position 3837) restriction sites (9). Samples of influenza A/WSN/33 for preparation of the NA-encoding cDNA insert in plasmid pT3NAm1 are obtainable, for example, from the W.H.O. Collaborating Centre, Division of Virology, National Institute for Medical Research, London, U.K.

An alternative plasmid which may be employed to construct DNA templates for transcription of mutant NA-encoding vRNA segments of influenza A/WSN/33 is the pUC19-derived plasmid pT3NAv, whose construction is described in WO91/03552 (Palese, P. *et al.*). Plasmid pT3NAv also contains the full length cDNA of the NA gene of influenza A/WSN/33 flanked by a promoter specifically recognised by bacteriophage T3 RNA polymerase and a restriction endonuclease cleavage site.

PCR products were made using pT3NAm1 as a template and the following primers modified to provide mutations as specified in Fig. 1:
5'-CGGAATTCGAAGACGCAGCAAAAGCAGGAGTTTAAATGAATCC-3' (primer 1) and 5'-
CCAAGCTTATTAACCCTCACTAAAAGTAGAAACAAGGAGTTTTTTGAA C-3' (primer 2) (the residues at which mutations were introduced are underlined, e.g. for construction of the D1 mutant cDNA, in both primers 1 and 2 the first underlined A nucleotide was substituted by a C nucleotide). The PCR products were digested

with EcoRI and HindIII restriction enzymes and they were cloned into pT3NAm1 cut with the same enzymes. NA genes and the flanking sequences in the modified plasmids were sequenced with an automated sequencer (Applied Biosystems).

The following double-mutations were introduced into the NA gene of influenza A/WSN/33 virus: U-A→G-C (10-11') (mutant D1), C-G→A-U (11-12') (mutant D2), and C-G→U-A (12-13') (mutant D3) (Fig. 1). In addition, six NA genes with the corresponding single-mutations were constructed (U→G10, A→C11', C→A11, G→U12', C→U12, and G→A13').

Example 2

Production of and transfection of ribonucleoprotein (RNP) complexes.

Transfectant viruses were prepared as described by Enami and Palese (5). NA-specific RNP complexes were reconstituted *in vitro* and transfected into MDBK cells infected with A/WSN-HK helper virus (5).

Synthetic RNAs were obtained by T3 RNA polymerase transcription of modified pT3NAm1 plasmids linearized with BbsI restriction enzyme. RNAs were reconstituted into RNP complexes using RNA polymerase and NP protein isolated from influenza X-31 virus. Influenza X-31 virus is a reassortant of influenza A/HK/8/68 and A/PR/8/34 viruses and was supplied by Evans Biological, Ltd., Liverpool, England. The RNP complexes were transfected by the DEAE-dextran transfection method into MDBK cells infected with WSN-HK helper influenza virus grown in 10-day embryonated chicken eggs. The MDBK cells were grown in reinforced minimal essential medium. For subsequent experiments, influenza A/WSN/33 wild-type virus was also grown in MDBK cells in reinforced minimal essential medium. Rescued transfectant viruses were plaque purified three times in MDBK cells. A single plaque was used for preparing a stock virus for further analysis.

Example 3

Sequencing of the NA genes of transfectant viruses.

The presence of the mutations in the transfectants was confirmed by sequence

analysis of the 3' and 5' terminal sequences of the NA gene. Viral RNA for sequencing was isolated by phenol-chloroform extraction from transfectant viruses purified by centrifugation through a 30 % sucrose cushion. In some cases, total RNA isolated with RNAzol B (Tel-Test, Inc., Friendswood, TX) from infected cells was used. Sequences of the 5' end were obtained either by direct RNA sequencing or by 5' RACE. Direct sequencing of the 5' ends was performed using a primer complementary to nucleotide positions 1280 to 1299 (5'-TGGACTAGTGGGAGCATCAT-3') of the influenza A/WSN/33 NA gene and an RNA sequencing kit (United States Biochemical Corporation, Cleveland, OH) following the manufacturer's instructions. For 5' RACE, viral RNA was reverse transcribed using a primer complementary to nucleotide positions 879 to 898 (5'-GGGTGTCCTTCGACCAAAAC-3') of the influenza A/WSN/33 NA gene. The reverse transcription product was extended with terminal deoxynucleotidyl transferase (TdT) (Gibco BRL, Gaithersburg, MD) and amplified by PCR with the primer used for direct RNA sequencing (see above) and the 5' RACE abridged anchor primer (Gibco BRL). PCR products, cut with SpeI restriction enzyme, were cloned into the XbaI site of pUC18 and sequenced with a DNA sequencing kit (United States Biochemical). In order to sequence the 3' end of the NA gene of transfectant viruses, viral RNA was 3'-polyadenylated using poly(A) polymerase (Gibco BRL). The polyadenylated RNA was reverse transcribed using the primer 5'-GCGCAAGCTTCTAGATTTTTTTTTTTTT-3' and the cDNA was amplified by PCR with a primer containing nucleotides corresponding to positions 115 to 98 (5'-GCGCAAGCTTTATTGAGATTATATTTCC-3') of the influenza A/WSN/33 NA gene and the primer used for reverse transcription. PCR products digested with HindIII were cloned into pUC18 and sequenced with the DNA sequencing kit.

Transfection of all three NA genes with double-mutations resulted in rescue of transfectant viruses (D1, D2, and D3). On the other hand, only three out of the six single-mutant constructs were rescued, carrying mutations at positions 10, 11', and 13' (Fig. 1). In three attempts, none of the other three constructs (with mutations at positions 11, 12, and 12') was rescued.

Confirmation of mutations in the two single mutant transfectants at positions

10 and 11' was more difficult since they were unstable. Specifically, cloning of the 3' end of the NA vRNA of the U-G10 mutant resulted in one clone with mutant and two clones with wild-type sequences. Direct RNA sequencing of the 5' end of the NA-specific vRNA from purified A-C11' transfectant, following three plaque to plaque passages, revealed a wild-type sequence. However, when NA-specific vRNA from MDBK cells infected with the original plaque of this transfectant was sequenced, the presence of the mutation was confirmed. Thus it seems likely that the transfectant reverted to wild-type during the plaque purification steps. This interpretation is supported by the observation that the transfectant initially produced small plaques, but showed larger plaques upon passaging. Taken together, sequencing data of the single mutants showed that transfectant viruses with single mutations, at least those with mutations at positions 10 and 11', are unstable.

Example 4

Growth properties of the D1, D2, D1/2 and D3 mutants

D1, D2, and D3 were grown on MDBK cells. Confluent monolayers of MDBK cells were infected at low m.o.i. (0.01) and the amount of infectious virus released into the medium was assayed at different time points by plaque assay on MDBK cells (Fig. 2). The D2 transfectant virus showed approximately one log reduction in plaque titre compared to the wild-type virus. However, D1 and D3 transfectant viruses were not significantly affected by the mutations. Consistently, the plaque size of D2 was reduced, but both D1 and D3 viruses showed plaque sizes similar to that of the wild-type.

The growth properties were also investigated of mutant influenza A/WSN/33 having multiple double-mutations in the NA-specific vRNA. A construct incorporating double-mutations from both D1 and D2 transfectants was successfully rescued (D1/2) (Fig. 1) into infectious virus. The D1/2 transfectant was plaque purified three times and the presence of mutations was confirmed by sequencing. This virus showed similar reduction in plaque titres (Fig. 2) and plaque size on MDBK cells as the D2 transfectant. The effect of the D1/2 mutations on viral growth was more dramatic on MDCK and Vero cells where reductions of at least three to

four logs in plaque titres were observed (see Examples 10 and 11 below).

Example 5

Measurement of NA levels in transfectant viruses

The level of NA expressed by the viruses was determined to see if it corresponded to growth levels. Influenza A/WSN/33 and transfectant viruses were grown in MDBK cells and purified by 30 to 60% sucrose gradient ultracentrifugation. About 10 μg of viral proteins were denatured with 0.5% SDS and 1% β-mercaptoethanol at 100 °C for 10 minutes and digested with 400 u of PNGase F (New England Biolabs, Inc., Beverly, MA) for 20 h at 37 °C in a reaction buffer containing 50 mM sodium phosphate, pH 7.5, 1% NP-40, and 5 mM Pefabloc (Boehringer Mannheim Corporation, Indianapolis, IN). The PNGase F treatment removes N-linked carbohydrate chains from NA and HA. This gives a better resolution of the NA band which migrates closely to NP and HA on gels. Proteins were analyzed by 12% SDS-PAGE and staining with Coomassie Brilliant Blue.

Both D2 and D1/2 virions showed a dramatic reduction in NA content compared to that of the wild-type virus or the D1 and D3 transfectants.

In order to quantitate NA levels of the D2 and D1/2 viruses, neuraminidase activity was measured. About 2 μg, 0.5 μg, 0.125 μg, and 0.031 μg (4 fold dilutions) of proteins from purified virus were incubated for 10 minutes at 37 °C in 150 mM phosphate buffer, pH 6.0, 1 mM $CaCl_2$, containing 50 nmols of 2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MU-NANA) as substrate in a total volume of 100 μl (27). Then 2 ml of stop buffer (0.5 M glycine/NaOH, pH 10.4) were added and the released 4-methylumbelliferone was determined by spectrofluorometry. 0.1 mM solution of 4-methylumbelliferone was used as a standard control. NA activity was expressed as nmoles of 4-methylumbelliferone released in 1 minute per μg of viral proteins.

NA activity associated with the wild-type virus was 2.18 nmol min^{-1} μg^{-1}. However, the transfectant viruses D2 and D1/2 exhibited only 0.24 and 0.25 nmol min^{-1} μg^{-1} activity, respectively. Thus, the transfectant viruses showed approximately a 10 fold reduction in NA activity compared to the wild-type virus which is in

agreement with the reduced NA levels observed in SDS-PAGE.

Example 6

NA-specific vRNA levels in purified transfectant viruses

Viral RNA from wild-type and transfectant viruses purified through a 30% sucrose cushion was extracted with phenol/chloroform. The viral RNAs purified from wild-type and transfectant viruses were analyzed by PAGE and the RNA segments were visualized by silver-staining. The NA segment was present in all transfectant viruses at levels comparable to that of the wild-type virus. In order to quantify NA-specific vRNA levels, a primer extension analysis was performed using vRNA extracted from purified viruses.

Primer extension analysis of NA and NS vRNA levels was performed as previously described (2). Briefly, 100 ng of viral RNA was transcribed with 200 u of SuperScript (Gibco BRL) for 1 h at 42 °C in the presence of 3×10^5 cpm of ^{32}P-labelled NA- and NS-specific primers. The NA-specific primer, 5'-GTGGCAATAACTAATCGGTCA-3', is complementary to nucleotides 1151 to 1171 of the NA vRNA. The NS-specific primer, 5'-GGGAACAATTAGGTCAGAAGT-3', is complementary to positions 695 to 715 of the NS vRNA. Primer extension reactions were stopped by adding an equal volume of 90% formamide and 10 mM EDTA followed by heating to 95 °C for 3 minutes. Extension products were analyzed on 5% polyacrylamide gels in the presence of 7 M urea and quantitated by phosphorimager analysis of dried gels (Molecular Dynamics).

The NS gene was used as an internal control. The amounts of NA-specific vRNA segments in the transfectant viruses were similar (±20%) to that of the wild-type virus in two experiments.

Example 7

NA-specific vRNA levels in cells infected with the D2 or D1/2 transfectant viruses.

MDBK cells were infected with wild-type or transfectant viruses at an m.o.i. of 2 and total RNA was isolated from cells at 3.0, 5.5, 8.0, and 10.5 h postinfection

with RNAzol B (Tel-Test). NA-specific vRNA levels in total RNA were measured by primer extension assay as described above in Example 6 using 5μg of total RNA. Cells infected with the D2 transfectant virus contained NA-specific vRNA levels similar (±10%) to those infected with the wild-type virus. Although cells infected with the D1/2 transfectant virus showed a 28 to 53% reduction in NA-specific vRNA levels (results obtained by phosphorimager analysis in two experiments at 5.5, 8.0, and 10.5 h postinfection), this decrease cannot account for the ten-fold reduction of NA protein levels.

Example 8

NA-specific mRNA and cRNA levels in cells infected with the D2 or D1/2 transfectant viruses.

Since NA-specific vRNA levels were not dramatically affected by the mutations in the D2 and D1/2 transfectant viruses, the 10 fold reduction in NA levels (see above) could result from a reduction in mRNA levels and/or from a defect in translation. In order to distinguish between these possibilities, the amounts of NA-specific mRNA in cells infected with D2 or D1/2 transfectant viruses were measured by using a primer extension assay. MDBK cells were infected at an m.o.i of 2 with wild-type or transfectant viruses and total RNA was isolated at 3.0, 4.5, 6.0, and 7.5 h postinfection.

Primer extension analysis of NA and HA mRNA and cRNA levels in total RNA from infected cells was performed under the same conditions as described in Example 6. The primer for NA-specific mRNA and cRNA, 5'-GCGCAAGCTTTATTGAGATTATATTTCC-3', contains 18 nucleotides (underlined) corresponding to positions 115 to 98 of the NA gene. The primer for the extension of HA-specific mRNA and cRNA, 5'-CATATTGTGTCTGCATCTGTAGCT-3', corresponds to positions 94 to 71 of the HA gene.

Since total RNA from infected cells contains both mRNA and cRNA, which differ only at their termini, signals for both species of RNAs were expected in the same primer extension assay. Due to the presence of a heterologous 10 to 15

nucleotides long capped primer at the 5' end of mRNA molecules, the signal for mRNA on gels appears as a multiple band containing DNA species of different sizes. On the other hand, the signal for cRNA appears as a single band, which is approximately 10 to 15 nucleotides shorter than the signal for mRNA.

NA-specific mRNA levels in cells infected with either D2 or D1/2 transfectant virus were below detection levels. NA-specific cRNA levels were apparently unaffected in these transfectant viruses. An additional band running slightly faster than the NA-specific cRNA band, detected in all samples, represents a nonspecific signal, since it was also detected in RNAs extracted from uninfected cells.

The observed attenuation of NA-specific mRNA levels in cells infected with the D2 transfectant is consistent with the previous findings of Kim *et al.* (14) that an A-U(11-12') base-pair mutation in the context of a vRNA-like CAT reporter gene resulted only in 22% reporter activity compared to a wild-type control. However, the G-C(10-11') and U-A(12-13') base-pair mutations, which had no effect on the expression levels of the neuraminidase of the D1 and D3 transfectants, resulted in only 20 and 31% activities, respectively, in a CAT reporter gene system (14). It is thus clear that base-pair mutations in the context of a CAT reporter gene system and a rescued native NA gene containing vRNA segment have different effects.

Example 9

In vitro transcription of NA-specific ribonucleoprotein complexes.

In theory, the reduction of mRNA levels observed as above could have been caused by a decrease in mRNA stability or by a decrease in mRNA synthesis. The interference with mRNA synthesis may occur at the point of initiation, e.g. capped RNA primer binding or endonuclease activity could be inhibited. Alternatively, termination or polyadenylation of viral mRNA could be affected. In order to distinguish between all these possibilities, *in vitro* transcription assays were performed.

Wild-type influenza A/WSN/33 virus, D2, and D1/2 transfectants were grown in MDBK cells and purified on a 30% sucrose cushion. Twelve 15 cm dishes were

used for each virus. The purified viruses were resuspended in 200 μl of PBS and disrupted by adding 50 μl of 5x disruption buffer (500 mM Tris-HCl [pH 7.4], 500 mM NaCl, 25 mM $MgCl_2$, 5 mM DTT, 25% glycerol, 2.5% NP-40, 2.5% Triton X-100, 50 mg ml^{-1} lysolecithin) and incubation at 37 °C for 30 min. The disrupted viruses were fractionated by centrifugation on a discontinuous glycerol gradient (70%, 50%, and 30%, 150 μl of each) in 100 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT. The gradients were centrifuged for 4 h at 15 °C in 0.8 ml tubes at 45,000 rpm in a Beckman SW55 rotor with adaptors. Fractions collected from the bottom of the tubes were analyzed by 12% SDS-PAGE and those enriched in RNPs were used in transcription assays.

In vitro transcriptional activity was measured using globin mRNA as primer. Transcription reactions were performed by using 6 μl of RNPs in a total reaction volume of 20 μl containing 50 mM Tris-HCl (pH 7.8), 50 μM KCl, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 0.5 mM each GTP and CTP, 50 μM UTP, 0.1 μM [α-^{32}P] UTP (3,000 Ci $mmol^{-1}$), 20 u of RNase inhibitor (Boehringer Mannheim Corporation, Indianapolis, IN), 0.6 μg of rabbit globin mRNA (Gibco BRL). After incubation at 31 °C for 1.5 h, transcription products were extracted with phenol/chloroform and precipitated in the presence of 5 μg of carrier yeast RNA.

NA-specific transcription products were synthesized from both the wild-type and the transfectant RNPs. However, there was a significant difference in the pattern of the bands. The wild-type NA-specific transcription product appeared as a wide band corresponding to RNA species with poly(A) tails of different sizes. On the other hand, the NA-specific transcription products of both the D2 and D1/2 transfectants produced less diffuse bands, which implied that these products might not be polyadenylated. In order to characterize the transcription products, they were analyzed by oligo(dT)-cellulose chromatography.

The fractions depleted of poly(A)-containing molecules showed higher levels of NA-specific transcription products for the D2 and D1/2 transfectants, but lower levels for the wild-type control. On the other hand, fractions enriched in poly(A)-containing molecules showed lower levels of the NA-specific transcription products for the D2 and D1/2 transfectants, but higher levels for the wild-type virus.

This seems to confirm that there is a large proportion of NA-specific transcription products of the D2 and D1/2 transfectants which lack poly(A) tails.

It is thus proposed that the mutations in the NA-specific vRNA of D2 and D1/2 interfere with polyadenylation of mRNA transcripts. The observed low levels of mRNA in cells infected with these viruses is fully consistent with this conclusion, since non-polyadenylated capped transcripts are most likely rapidly degraded in the cell (30).

Example 10

Growth of transfectant viruses on MDCK cells.

MDCK cells in 96-well plates were infected with $5x10^4$ pfu and 10 times dilutions of wild-type influenza A/WSN/33 virus, or transfectant D1, D2, D3, and D1/2 viruses. Four wells were used for each virus. Infected cells were maintained in 100 μl of Dulbecco's minimal essential medium (DMEM) supplemented with 10% bovine serum albumin and 1 μg/ml of trypsin. After 72 h, 50 μl of the medium was tested for hemagglutination with 50 μl of 1.5% red blood cells and ID_{50} was calculated for each virus. ID_{50} is defined as the dose at which 50% of the medium of the infected cells gives a positive haemagglutination signal. It was found that the ID_{50} for the wild-type virus and the D1 transfectant was 5 pfu. On the other hand, the ID_{50} of the D3 transfectant was 20 times higher. The ID_{50} of the D2 and D1/2 transfectant was approximately 3000 times higher than that of the wild-type or the D1 transfectant.

Example 11

Growth of the D1/2 transfectant on Vero cells.

Confluent Vero cells in 35 mm dishes were infected at an m.o.i. of 0.01 with wild-type influenza A/WSN/33 virus or D1/2 transfectant in duplicates. Cells were maintained in DMEM supplemented with 2% FBS for 72 h and virus present in the medium was titrated by plaque assay on MDBK cells. The wild-type virus reached $5x10^7$ pfu/ml, but there was less than $5x10^2$ pfu/ml of infectious virus in the medium from the cells infected with the D1/2 transfectant.

Taken together, the data in Examples 4, 10 and 11 show that base-pair mutations in the double-stranded region of the promoter of an influenza A virus vRNA can lead to reduced growth of influenza virus in tissue culture. As noted above, the D2 and D1/2 transfectant viruses showed approximately one log reduction in growth in MDBK cells, while both the D1 and D3 viruses grew like the wild-type. A more dramatic reduction in growth was observed for the D2 and D1/2 viruses on MDCK and Vero cells. Interestingly, the D3 transfectant showed reduced growth on MDCK cells compared to the wild-type. Both D2 and D1/2 transfectants exhibited approximately four log reduction on MDCK cells, and the D1/2 transfectant 5 log reduction on Vero cells. Such results are indicative that influenza A viruses having the D2 and D1/2 mutations will exhibit effective attenuation *in vivo*.

Example 12

Passage of transfectant viruses and sequencing to determine the stability of the D1, D2 and D3 mutations

Stocks of D1, D2, and D3 transfectant viruses with confirmed double-mutations were plaqued on MDBK cells and individual plaques were passaged ten times on MDBK cells at a low m.o.i. After ten passages, the viruses were plaqued and single plaques were used to prepare virus stocks for sequencing. Stocks of passaged viruses were purified through a 30 % sucrose cushion and viral RNA was isolated by phenol-chloroform extraction. In order to sequence the 3' end of the NA gene, viral RNA was 3'-polyadenylated using poly(A) polymerase (Gibco BRL, Gaithersburg, MD). The polyadenylated RNA was reverse transcribed using the primer 5'-GCGCAAGCTTCTAGATTTTTTTTTTTTTT-3' and the cDNA was amplified by PCR with a primer containing nucleotides corresponding to positions 115 to 98 (5'-GCGCAAGCTTTATTGAGATTATATTTCC-3') of the influenza A/WSN/33 NA gene and the primer used for reverse transcription. PCR products digested with HindIII were cloned into pUC18 and sequenced with a DNA sequencing kit (United States Biochemical, Corporation, Cleveland, OH).

Three clones originating from three individually passaged plaques of the D1 transfectant showed the presence of the U~G10 mutation. All clones obtained from 5

individually passaged plaques of the D2 transfectant had the expected C→A11 mutation. In addition, two of the clones showed a U→C change at position 4 which is a natural variation observed among different influenza A virus isolates. In two of the clones, we have also found a U→C mutation at position 23 adjacent to the initiation codon for the neuraminidase which changes the second amino acid of NA from an asparagine to an aspartate. Only two of the clones obtained from the D3 transfectant showed the C→U12 mutation. The third clone had a wild-type sequence indicating that this base-pair mutation might not be stable. A reversion of A→G13' could result in a viable virus with a U-G(12-13') base-pair, which could then revert to the wild-type C-G(12-13') base-pair by a U→C12 change. Due to the presence of different residues such a reversion cannot occur at the other two studied base-pairs.

In summary, the mutations in the 3' end of D1 and D2 transfectants were preserved during ten passages. Preliminary data confirms the presence of the mutations also in the 5' end of the NA segment of the passaged transfectant viruses. It can be assumed that transfectant viruses with double-mutations should be stable since two specific mutations would have to occur simultaneously in order to revert to the wild-type sequence. It did not prove possible to rescue any transfectant viruses with C→A11 or G→U12' single mutations which suggests that such viruses might be severely impaired or not viable at all.

Example 13

Attenuation of D2 and D1/2 viruses in mice

Influenza A/WSN/33 wild-type and transfectant viruses D1, D2, D3 and D1/2 were grown at 37°C in Madin-Darby bovine kidney (MDBK) cells in reinforced minimal essential medium. Plaque assays were performed on MDBK cells.

Groups of five female BALB/c mice were used for influenza virus infection at 6 to 12 weeks of age. Intranasal (i.n.) inoculations were performed in mice under ether anesthesia using 50μl of PBS containing 10^6, $3x10^4$ or 10^3 plaque forming units (pfu) of D1, D2, D3 or D1/2 virus. As controls, mice were infected with wild-type influenza A/WSN/33 virus using the same pfu of virus. This virus was rescued by ribonucleoprotein transfection of a wild-type NA gene as previously described by

Enami and Palese (4). Animals were monitored daily and sacrificed when observed in extremis. All procedures were in accord with NIH guidelines on care and use of laboratory animals. The results are shown in Figures 4 to 9.

All mice infected with wild-type virus developed signs of disease and died by day 15 post-infection. However, all mice infected with the D2 or D1/2 viruses survived. Only those D2 or D1/2 virus-infected animals lost weight which were infected with the high dose of virus (10^6 pfu); they lost 10 to 20% of body weight by day 3 post-infection, but they quickly recovered in the following days. The virulence of the D1 virus was indistinguishable from the virulence of wild-type virus in these experiments. The D3 virus showed a slightly attenuated phenotype in mice.

Example 14

Impaired replication of the D2 and D1/2 viruses in mouse lungs

Groups of 6 BALB/c mice were infected intranasally as above with 10^3 pfu of wild-type, D1, D2, D3 or D1/2 viruses. Three days post-infection, three mice per group were sacrificed, their lungs were extracted and homogenized in 2 ml of PBS, and virus titres were measured by plaque assay in MDBK cells. Six days post-infection, the rest of the mice were also sacrificed and viral titres were determined in their lungs by the same protocol. The results are shown in Fig. 10.

The wild-type and the D1 viruses grew to high titres in the lungs of the infected mice (approximately 10^6 and 10^7 pfu/ml at days 3 and 6 post-infection, respectively). Titres in the lungs of mice infected with the D3 virus were approximately one and a half logs lower. By contrast, viral titres were not detectable or very low (less than 10^3 pfu/ml) in the lungs of the D2 or D1/2 infected mice. The results demonstrate that replication of the D2 and D1/2 viruses is highly impaired in mouse lungs.

Example 15

Induction of protective immunity by D2 and D1/2 viruses

Sera from the groups of surviving mice which were intranasally infected with D2 or D1/2 virus as above was collected and pooled 3 weeks after infection. The

sera were treated with receptor destroying enzyme (Sigma) to eliminate unspecific inhibitors of influenza virus-mediated haemagglutination as previously described by Burnet and Stone (55). The haemagglutination inhibition (HI) titres were determined as the highest serum dilution that was able to neutralize the haemagglutination activity of a preparation of influenza A/WSN/33 virus with an HA titre of 8. In these assays, 0.5% chicken red blood cells were used.

All pools of sera which were tested were found to contain antibodies against influenza A/WSN/33 virus with HI activity. HI titres were higher in the animals immunized with the higher virus doses (see Table 1 below).

In addition, all mice which were intranasally infected with D2 or D1/2 virus were observed to be protected against death and disease (as measured by body weight loss) when challenged with a lethal infection dose (more than 1000 LD_{50}s) of wild-type A/WSN/33 virus (see Table 1 and Figures 11 and 12).

Table 1

Protection against wild-type influenza virus infection in mice immunized with D2 and D1/2 viruses

Immunizing virus	Immunizing dose	HI titres	Challenge: 10^6 pfu of wild-type virus Number of survivors
D2	10^6 pfu	352	5/5
	3 x 10^4 pfu	160	5/5
	10^3 pfu	24	5/5
D1/2	10^6 pfu	160	5/5
	3 x 10^4 pfu	44	5/5
	10^3 pfu	72	5/5

Example 16

Use of the D1/2 transfectant virus as a helper virus to rescue NA genes

As noted above, the D1/2 transfectant virus showed approximately 5 log reduction in growth on Vero cells compared to wild-type influenza A/WSN/33. It can therefore be employed to provide an alternative rescue system for rescue of

NA-encoding vRNA segments of influenza A viruses. An appropriate protocol for this consists of the following steps:

1. infection of MDBK cells with D1/2 helper virus;
2. treatment of the infected MDBK cells with DEAE-dextran/DMSO transfection reagent;
3. transfection of a synthetic NA ribonucleoprotein complex into D1/2 helper virus infected and DEAE-dextran/DMSO-treated MDBK cells; and
4. selection of rescued viruses on Vero cells.

Only viruses which acquire the transfected NA gene grow to high titre on Vero cells.

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